Airway Metabolic Anomalies in Adolescents with Bronchopulmonary Dysplasia: New Insights from the Metabolomic Approach

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Objectives To assess a group of adolescents with bronchopulmonary dysplasia (BPD) from a biochemical-metabolic standpoint, applying the metabolomic approach to studying their exhaled breath condensate (EBC).

Study design Twenty adolescents with BPD (mean age 14.8 years) and 15 healthy controls (mean age 15.2 years) were recruited for EBC collection, exhaled nitric oxide measurement, and spirometry. The EBC samples were analyzed using a metabolomic approach based on mass spectrometry. The obtained spectra were analyzed using multivariate statistical analysis tools.

Results A reliable Orthogonal Projections to Latent Structures-Discriminant Analysis model showed a clear discrimination between cases of BPD and healthy controls ($R^2 = 0.95$ and $Q^2 = 0.92$). The search for putative biomarkers identified an altered complex lipid profile in the adolescents with BPD.

Conclusions The metabolomic analysis of EBC distinguishes cases of BPD from healthy individuals, suggesting that the lung of survivors of BPD is characterized by long-term metabolic abnormalities. The search for putative biomarkers indicated a possible role of an altered surfactant composition, which may persist far beyond infancy. *(J Pediatr 2015;166:234-9)*.

See editorial, p 224

Bronchopulmonary dysplasia (BPD) is the main cause of respiratory morbidity in prematurely born babies. After the acute phase of the disease, survivors of BPD often improve in their respiratory symptoms during infancy and early childhood, but many have airflow limitations that persist into adolescence and adulthood.[1]

Although many studies report the long-term functional outcome of subjects with BPD, very little has been published about the pathologic mechanisms involving the lungs of survivors of BPD. Some recent data suggest that prematurity may be associated with persistent neutrophilic airway inflammation and oxidative stress in later life.[2,3]

In this study, we investigate the pathologic mechanisms behind BPD by using metabolomic approach applied to exhaled breath condensate (EBC). EBC is a biofluid collected noninvasively by cooling the air expired during tidal breathing.[4] EBC is easy to collect, and the condensate can be analyzed to assess metabolic activity and study active pathophysiological processes in the lung.[4]

Metabolomics is a high-dimensional biological data analysis method that identifies the small molecules generated by cellular metabolic activity. Being guided by no a priori hypothesis, the metabolomic approach simultaneously considers a large number of metabolites in a biological sample and allows for their representation in a spectrum. Then, bioinformatic tools for multivariate data analyses are applied to identify metabolite profiles, or metabolic fingerprints, that may enable subjects with disease to be characterized and distinguished from healthy controls.[5,6]

Metabolomic analysis was used to study exhaled breath and urine in asthma and chronic obstructive pulmonary disease,[7-10] demonstrating that this approach may help us to discover disease-related biomarkers.

The aim of the present study was to apply metabolomic analysis as an unbiased approach to noninvasively investigate the respiratory tract of survivors of BPD from a biochemical-metabolic standpoint. We compared EBC from cases of BPD and healthy controls to identify whether

BPD	Bronchopulmonary dysplasia	LPC	Lyso-phosphatidylcholine
DECCS	Disposable exhaled condensate collection systems	OPLS-DA	Orthogonal projections to latent structures-discriminant analysis
EBC	Exhaled breath condensate	PAF	Platelet activating factor
FE_{NO}	Fractional exhaled nitric oxide	pred	Predicted
FEV1	Forced expiratory volume in 1 second	RT	Retention time
FVC	Forced vital capacity	TURBO	Transportable unit for research on biomarkers

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Supported by the Italian Ministry of Health (Giovani Ricercatori 2009-1604820). The authors declare no conflicts of interest.

http://dx.doi.org/10.1016/j.jpeds.2014.08.049

the obstructive respiratory pattern of BPD is associated with a specific biochemical-metabolic EBC profile suggestive of ongoing pathological processes.

Methods

We recruited 20 consecutive survivors of BPD who were routinely followed up at our Unit of Pediatric Respiratory Medicine. They had been admitted to the Neonatal Intensive Care Unit at the Pediatrics Department (University of Padova, Italy) between January 1987 and December 1994. BPD was defined as oxygen dependence persisting for 28 days after birth. At birth, their mean gestational age was 28.4 weeks (SD 1.6), and they weighed a mean 1028 g (SD 242). At recruitment for the present study, the mean age of the subjects with BPD was 14.8 years.

All the survivors of BPD were clinically stable at recruitment; 5 of them were on low- to medium-dose inhaled steroid treatment associated with a long-acting beta-agonist; 1 was being treated with montelukast. Three of the survivors of BPD treated with inhaled steroid and long-acting beta-agonist were sensitized to common airborne allergens (dust mites, dog dander, grass, and tree pollens).

Age- and sex-matched healthy adolescents (n = 15; mean age of 15.2 years) with no history of preterm birth, atopy, or respiratory diseases were recruited from a public school as a control group.

None of the participants had any doctor-diagnosed lower airway infection in the previous 4 weeks, and they reported no signs of upper respiratory tract infection for the past week.

At recruitment, all the participants had a physical examination and a fractional exhaled nitric oxide (FE_{NO}) measurement, spirometry, and EBC collection. The EBC was then stored at −80°C until it was analyzed. The ethics committee at our hospital reviewed and approved the protocol (Prot. N 1360P), and all parents gave their informed consent.

EBC Collection

EBC was collected and processed according to the American Thoracic Society/European Respiratory Society recommendations.[4] The transportable unit for research on biomarkers from disposable exhaled condensate collection systems (TURBO DECCS) (Medivac, Parma, Italy) was used for EBC collection.[11] The TURBO is a refrigerating system based on a thermo-electrical module that generates a Peltier effect. The cold side of the Peltier module is connected to an aluminium holder shaped to house the test tube. The TURBO is supplied together with the DECCS, disposable respiratory systems that consist of a mouthpiece fitted with a 1-way valve and a reliable saliva trap, connected to a collecting vial (50 mL) by means of a tube. Participants breathed tidally through the mouth for 20 minutes, while sitting comfortably and wearing a nose clip. They kept their mouth dry during EBC and periodically swallowed excess saliva. EBC samples were immediately stored at −80°C in polypropylene tubes until assay. Orbitrap liquid chromatography mass spectrometry analysis of metabolites in EBC is available in the **Appendix** (available at www.jpeds.com).

FE_{NO} and Lung Function Measurement

FE_{NO} was measured with the NIOX system (Aerocrine, Stockholm, Sweden) using a single-breath on-line method according to the ERS/ATS guidelines for measuring FE_{NO} in children.[12] Lung function was measured with a 10-L bell spirometer (Biomedin, Padova, Italy).

Statistical Analyses

Spectroscopic data from mass spectrometry were analyzed using multivariate projection methods. As a first step, principal component analysis was applied for pattern recognition purposes.[13] The cluster structure highlighted was investigated by building discriminant models based on orthogonal projections to latent structures-discriminant analysis (OPLS-DA).[14,15] OPLS-DA is a supervised classification technique suitable for parsimonious classification models where the information useful for discriminating the 2 groups of subjects under investigation (in our study cases of BPD and healthy subjects) is summarized in only 1 predictive latent variable resulting from a linear combination of the measured variables. Permutation tests on the responses and n-fold cross-validation procedures with different values of n were run to check the validity of the model.[16,17] To explain the difference between the groups in terms of single measured variables, we built the so-called S-plot, where the covariance and the correlation between the measured variables and the predictive latent variable are shown in the same plot. A procedure based on random permutation allowed the estimation of the CIs useful to select the putative biomarkers during the inspection of the S-plot (confidence level equal to 95%).[18]

The measured variables underwent median fold change normalization and were log-transformed.[19] Pareto scaling with mean centering was applied before performing multivariate data analysis. Multivariate statistical data analysis was performed using the platform SIMCA 13 (Umetrics, Umea, Sweden), and the platform R 2.15.0 (R Foundation for Statistical Computing) was used for statistical analysis on single variable.

The discriminating compounds were identified by comparing the mass spectra with data from the METLIN (http://metlin.scripps.edu/xcms), Human Metabolome Database (http://www.hmdb.ca), and LIPID MAPS (http://www.lipidmaps.org). The search was conducted using a 10 ppm mass accuracy and only $[M+H]^+$ as molecular ion species. The clinical data (spirometric variables and log-transformed FE_{NO} values) were analyzed using the *t* tests for unpaired data.

Results

The mass spectrometry analysis on the EBC samples generated spectra in which the metabolites were represented according to their mass to charge ratio. The information